PHYSIOLOGY OF REPRODUCTION OF OSTREA VIRGINICA

II. Stimulation of Spawning in the Female Oyster

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Introduction

The second part of the investigations on the physiology of reproduction of the oyster (see Galtsoff, 1938) deals with the conditions which provoke ovulation and the discharge of eggs by ripe females. The work was carried out at the U. S. Fisheries Laboratory at Woods Hole. Live material for the experiments was obtained from Onset and Wellfleet, Massachusetts; Long Island Sound and the Chesapeake Bay. No difference was observed in the reactions of oysters from these three localities.

Ecological literature provides numerous data suggesting a correlation between the breeding periods of various marine animals and sea temperature (Plymouth Marine Invertebrate Fauna, 1904; Lo Bianco, 1909; Zernov, 1913). An attempt to analyze a mass of these observations from a physiological point of view was made by Orton (1920), who grouped all marine organisms into three classes: (*a*) those which breed at a definite temperature characteristic for the species throughout its range of distribution; (*b*) those in which breeding is influenced by a change in temperature, either at the maximum or the minimum for the locality; and (*c*) those which breed the year round. Yonge (1930) has demonstrated that some of the animals of the latter group breed for the greater part of the year not continuously, but periodically. Numerous observations on *O. virginica* (Ryder, 1887; J. Nelson, 1904; Moore, 1907; Churchill, 1920; Gutsell, 1924; Prytherch, 1928; Galtsoff, Prytherch and McMillin, 1930) have shown that this species spawns at rising temperature and therefore should be included in the second group of Orton's classification. Temperature about 20° C. was considered by these observers to be the lowest at which spawning takes place. Nelson (1928*b*) states that "once this 'critical temperature,' 20° C., is reached a 'trigger' mechanism is released which requires some hours for its consummation, and that the speed of the underlying processes is governed by temperature." Ecological evidence upon which this conclusion is based consists in daily records of the occurrence of oyster larvae in plankton, temperature data and weekly examination of oyster gonads. Its inadequacy in dealing with the physiological problem is apparent for neither the act of spawning nor the exact temperature of water, which is supposed to induce it, were observed in the field. Because the daily fluctuations in temperature in shallow water may exceed several degrees, the concept of "critical" temperature in nature has very little meaning unless it is specified whether the daily average or maximum or minimum is considered. Further observations by Nelson (1931) made in England show that *O. virginica* can be induced to spawn at 19.1° C. This throws further doubt on the validity of the view that 20° C. is the "critical" temperature in spawning of this species.

That temperature is not the only factor which governs the propagation of the oyster is shown by the observations of Hopkins (1931) and Loosanoff (1932) in Texas and Virginia. The breeding season in these waters extends over a period of several months during which the temperature remains well above 20° C. Since outbursts of spawning occurring during this time do not coincide with the rise or fall of temperature, other stimuli must be responsible for provoking sexual activity. Inasmuch as field observations fail to throw light on their nature, an experimental investigation conducted under carefully controlled laboratory conditions appears to be more promising.

Stimulation by Temperature

The question whether temperature alone can stimulate ovulation and the discharge of eggs from the pallial cavity of an oyster was studied in the two sets of laboratory experiments designed to determine the effects (1) of a prolonged exposure at increased constant temperature, and (2) of a quick rise of temperature from about 20° C. to 34°–35° C.

In the first series of experiments ripe females, kept in a live car in the bay, were brought to the laboratory and placed in water warmed by means of a no-lag immersion heater, to a desired degree. By using a mercury thermoregulator the temperature was kept constant within $\pm$ 0.2°. To avoid the effect of chemical stimulation by sperm which might have been present in the laboratory water supply, all the water was filtered through a 2½ inch layer of asbestos. If the experiments lasted more than 12 hours, running sea water was provided, otherwise the water was aerated and stirred but not changed. The beginning and end of the spawning reaction were determined from the kymograph records of shell movements. The presence of eggs which were collected from the bottom of the tank and kept under observation for

24 hours served as a supplementary proof that spawning took place. In all the cases they were unfertilized and failed to develop, indicating the absence of sperm in the water. The time which elapsed from the moment the oyster was exposed to a given temperature until the beginning of spawning is designated as the latent period of the reaction. The temperature of water in which the oysters were kept before the experiments varied between 19.5° and 20.6° and the range of temperatures to which they were exposed during the experiments extended from 22.6° to 30.0° C. For the sake of convenience the results presented in Table I are arranged in four classes beginning with a temperature increase not exceeding 2.5° and ending with a ten degree rise. Many experiments in which oysters failed to respond to temperature stimulation are not included in this table, which contains only positive results. An examination of column 6 discloses the lack of correlation between the temperature and the latent periods of spawning reactions. Each class of experiments comprise two distinct groups of slow and

TABLE I

Stimulation of spawning by temperature

Experiment number	Date	Temperature ° C.			Latent period	F
		Before	During	Increase		
					minutes	*hrs.*×° *C.*
437	July 9, 1934	20.6	22.5	1.9	591	19.7
437a	July 9, 1934	20.6	22.5	1.9	659	20.87
399	July 15, 1932	20.0	22.5	2.5	3060	127.50
498	July 5, 1935	20.3	25.0	4.7	35	2.74
498a	July 5, 1935	20.3	25.0	4.7	12	0.94
317	July 7, 1931	19.9	25.3	5.4	250	22.50
433	July 7, 1934	21.4	25.4	4.0	501	33.40
433a	July 7, 1934	21.4	25.4	4.0	500	33.30
321	July 9, 1931	19.9	25.0	5.1	22	1.87
325	July 10, 1931	19.5	24.5	5.0	65	5.42
492	Sept. 5, 1934	20.4	26.0	5.6	71	6.63
432	July 6, 1934	21.0	27.5	6.5	338	36.62
432a	July 6, 1934	21.0	27.5	6.5	340	38.83
318	July 8, 1931	19.9	28.0	8.1	32	4.32
319	July 8, 1931	19.9	28.5	8.6	55	7.88
322	July 9, 1931	19.9	26.0	6.1	257	26.13
320	July 8, 1931	19.9	30.0	10.1	42	7.07
438	July 10, 1934	20.6	30.0	9.4	405	63.45
438a	July 10, 1934	20.6	30.0	9.4	450	70.50
340	July 17, 1931	20.4	30.0	9.6	20	3.20

quick reacting oysters, the latent periods of which at 25° and above, fall either within 12 to 71 or between 250 and 501 minutes. Three experiments carried at 22.5° C. also show two groups of oysters with latent periods of about 600 and 3,000 minutes respectively. By examining the data presented in column 6 of the table, one can notice that the values of latent periods in different groups almost overlap. For instance, the minimum latent period of the first group (22.5°) is 591 minutes as compared with 501 minutes, the maximum latent period of the next higher temperature group. Plotting the latent periods against the temperature results in an erratic distribution of data showing an absence of any definite trend. Similar results are obtained if the amount of heat, *F*, received by the oyster and expressed as a product of the latent period multiplied by the degrees of increase in temperature, is considered. Examination of the last column of Table I shows wide fluctuation in this value and the absence of any definite correlation between it and the latent period.

Since under the conditions of the experiments, negative results have no meaning unless it is proved that experimental oysters were capable of spawning, every female that failed to respond to the increase of temperature was at the end of the experiment stimulated by sperm. Positive reaction was a proof that the female was physiologically ripe and that the lack of the response was due to the ineffectiveness of the stimulus. It was found that thermic stimulation fails in a larger number of ripe females. For instance, of the two females used in experiment No. 399, only one (shown in Table I) spawned at 22.5° C. Thirty-two hours of exposure at this temperature failed to stimulate spawning in the other one, which, however, copiously discharged eggs within a few minutes after the addition of sperm. In another experiment (not listed in Table I) three ripe females were taken from the tank in which the temperature during the previous week fluctuated between 18.5° and 19.5° C., and were placed in filtered sea water. The temperature was maintained at 22.6° but occasionally rose for a short time to 23.4°. Continuous kymograph records of shell movement of each of the females were obtained. The first oyster was kept under observation for 5 hours, 22 minutes; the second for 29 hours, 14 minutes; and the last one for 73 hours, 13 minutes. None of the oysters spawned during the experiment but all three shed eggs after sperm was added to the water (22.6°), the latent period being 16, 24, and 15 minutes respectively (Galtsoff, 1932). During the course of the investigations a large number of similar cases were recorded. Undoubtedly some of the oysters which were kept only for 24 hours at a constant temperature might have spawned if the experiments had been continued for a longer period of time. This was, however, impractical and the majority of the experiments had to be completed within one day. The percentage of ripe females which under those conditions were not responsive to temperature stimulation remains therefore undetermined. Yet the fact that within a range from 22.5° to 30° spawning in some of the females can be stimulated by exposing them to a constant temperature, higher than that of their original environment, is well established.

In a second set of experiments carried out in the summer of 1937, the effect of rapid rise in temperature from about 20° C. to 34°–35° C. was studied. After the oyster was placed in the experimental tank the water was quickly heated by an electric Hotpoint immersion heater, which was immediately removed as soon as the thermometer had registered the desired temperature. Then the water was allowed to cool. Usually it required about 20 minutes to bring the temperature from 20° to 34° C. In several instances oysters spawned before 34° had been reached. Out of 43 females used in these experiments, 17 or 39.5 per cent were stimulated to spawn; 24 or 55.8 per cent failed to react to temperature but were induced to spawn by the addition of sperm; and 2 or 4.7 per cent failed to respond to the combined action of both stimuli. The latent periods of the reactions (Table II) i.e., time elapsed between the moment the heat was turned on and the beginning of spawning, varied from 17 to 48 minutes. One can notice that the lowest average latent period occurs at the temperature between 33° and 33.9° C. On account of a small number of observations it is, however, impossible to state whether the observed differences have physiological significance and the temperature between the 33° and 34° is the optimum for stimulation of spawning in the female. At 35° and above, the adductor muscle contracts and the shell remains closed. With the drop of temperature to 34° the adductor relaxes again. No spawning was observed above 35° and oysters exposed for two minutes to temperatures between 35° and 37° and left after that

TABLE II

Latent periods (in minutes) of spawning reactions stimulated by rapid rise of temperature

Temperature		Latent period			Number of observations
Before spawning	During spawning	Maximum	Minimum	Average	
18–21	32.0–32.9	40	22	33	3
18–20.5	33.0–33.9	20	20	20	2
18–22.5	34.0–34.9	48	17	25.5	12

for thirty minutes at 32° to 33° failed to spawn but immediately discharged eggs upon the addition of sperm.

Sudden rise of temperature from 20° to 32°–35° appears to be a more effective stimulant than long-continued exposure to lower constant temperatures. The question arises whether the high temperatures used in these experiments occur in nature and are therefore significant in the propagation of the species. It is true that in northern waters the bottom temperature of the estuaries rarely reaches 27°–28° C. On the other hand, temperatures above 30° often occur in the coastal waters of the southern states. Hopkins has shown (1931) that in June–August the temperature over the oyster beds in Offats Bayou (Texas) remained above 30° and often exceeded 33° C. And further observations in other parts of the Texas coast, made by the Bureau of Fisheries in 1935, confirmed his findings. Furthermore, oysters exposed at low tide to direct sun rays are often heated to a much higher degree than that used in the experiments. For instance, temperatures from 29.2° to 35.8° were recorded by the author in the tissues of oysters exposed during a hot summer day above low water mark on tidal flats of Milford Harbor (Connecticut) while the water temperature in small tidal pools varied from 26.5° to 37.2°. Thus the temperature to which oysters were subjected in the laboratory lies within the thermal range of their normal habitat and the conditions of the experiment cannot be considered abnormal or even unusual.

Stimulation by Sperm

The sperm suspension used in all the experiments was prepared by mincing and squeezing 1 gram of ripe gonad tissue in a small amount of filtered sea water and diluting it up to 100 ml. Amounts varying from 0.03 to 5 ml. of this standard suspension were added to 30 liters of water in which the female oyster was kept. Mechanical stimulation was avoided by emptying the pipette in the vicinity of the stirrer, several inches from the body of the oyster. The water was agitated, aerated, and kept at constant temperature. This technique was followed throughout the investigation with the exception of a few special experiments in which sperm suspension was injected directly into the pallial cavity of the oyster.

It was shown in a previous paper (Galtsoff, 1930) that in a physiologically ripe female live sperm initiates a series of reactions which culminate in ovulation and rhythmical contractions of the adductor muscle. The first noticeable reaction of the oyster consists in one or several sharp contractions of the adductor usually within a few seconds after the addition of sperm (Fig. 1). This can be regarded as an ejection reaction caused by a chemical stimulation of the neural receptors of the tentacles and mantle. The presence or absence of this reaction is not a sign of the readiness of the female to spawn for the spawned-out and unripe oysters react in a similar manner and furthermore identical response can be obtained with any chemical substance to which the neural receptors of the oyster are sensitive. It is certainly not a part of the complex spawning reaction of the female.

The approach of spawning is manifested by the relaxation of the adductor muscle and decrease in the frequency of muscular contractions. The latent period during which these changes occur can be accurately measured from the kymograph records for the beginning of the spawning reaction is unmistakably marked by a series of rapid rhythmical contractions and the maintenance of a definite tonus level (Fig. 1).

The latent period determined in 101 experiments varies from 6 to 38 minutes. The frequency distribution of latency (Fig. 2) plotted for all these experiments, carried in different years and at temperatures ranging from 22.6° to 30.0° C., indicates the predominance of periods lasting from 9 to 12 minutes. In 78 per cent of the observations the latent period is less than 15 minutes. Its average value computed from all the records is 16.2 minutes. The latent period is independent of the concentration of sperm used in stimulating the reaction. This fact becomes apparent by examining Fig. 3 in which the latency indicated by ▲, is plotted against concentration. The present results confirm previous observations of Galtsoff (1930) in which no significant correlation was found between the two variables (Coef. of cor. 0.093 ± 0.106).

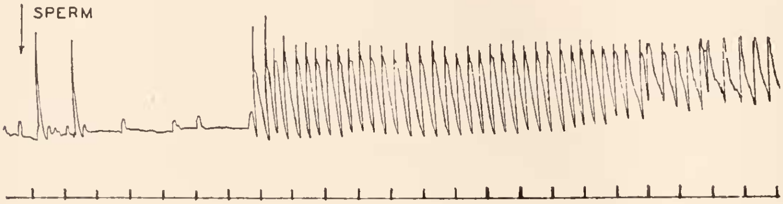

FIG. 1. Stimulation of spawning reaction in female *Ostrea virginica* by sperm. Time interval, 1 minute. Temperature 22.6° C.

There is possibly a slight indirect correlation between the length of the latent period and temperature. Between 25.1° and 30° the peak of the frequency distribution (Fig. 2, blocked-in area) is between 6 and 9 minutes and average value of latent period (22 observations) is only 14.3 minutes as compared with the average of 16.7 minutes and the peak between 9 and 15 minutes at a temperature from 20.1° and 25°

(cross-hatched areas, 79 observations). Both groups, however, cover the entire range of variations.

The next problem deals with the effect of the concentration of sperm on intensity of spawning. If the stimulating effect is due to the direct action of spermatozoa on the receptors of the female one would expect that both the latent period and the intensity of spawning should vary in proportion to the intensity of the stimulus, i.e., concentration of sperm. The duration of spawning reaction can be taken in this case as a measure of the intensity of the response. There is, however, no correlation between the two sets of data, as can be seen from Fig. 3, in which the duration of the reaction is indicated by □. Both the duration of the spawning reaction and its latent period are independent of the intensity of the stimulus.

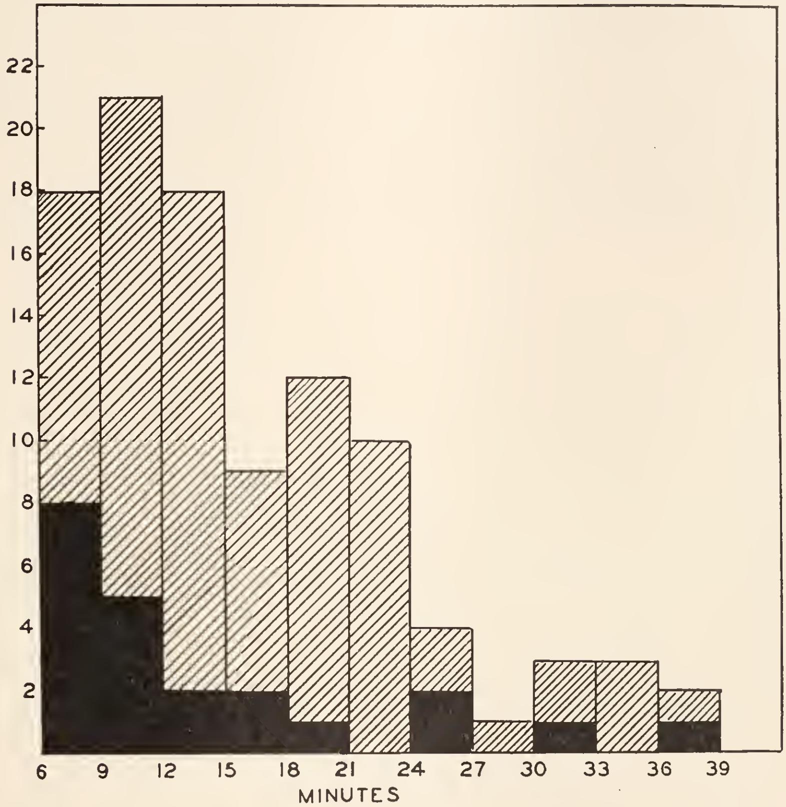

FIG. 2. Frequency distribution of the latent periods of spawning reaction in *Ostrea virginica*. Observations at temperatures between 25.1° and 30° C. are indicated by solid black areas; at 22.5–25.0°, by cross-hatched areas.

The determination of the threshold of sensitivity of the ripe female to the presence of sperm was made by adding gradually increasing amounts of standard sperm suspension to the known volume of water in which the females were kept. The minimum dose of sperm capable of inducing spawning in the female (Fig. 4) was found to be between 0.02 and 0.03 ml. added to 30 liters of water. Counts made by means of a haemocytometer showed that the suspensions used in two experiments contained from 100 to 156 millions of live spermatozoa per 1 ml.

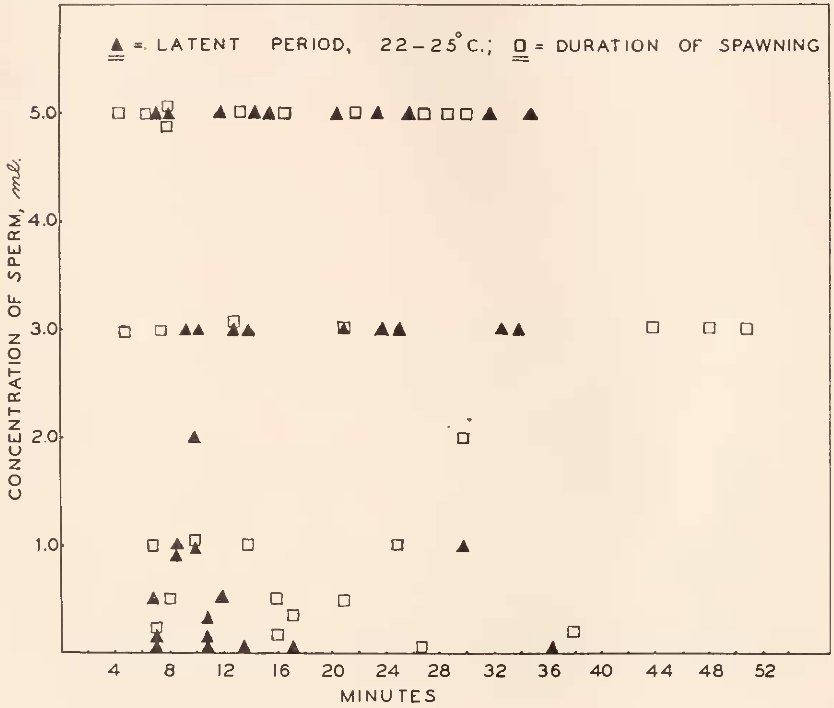

FIG. 3. Independence of latent periods and duration of spawning reaction in *Ostrea virginica* of the concentration of sperm.

or from 100 to 150 (in round figures) per milliliter of water surrounding the oyster. Since in other experiments the sperm suspension was standardized by weight, the exact figures of the number of spermatozoa per ml. probably have no definite significance, especially in view of the fact that the number of other cells present in suspension may vary depending on the state of the gonad development. Furthermore, oysters with greater pumping capacity may filter larger amounts of water and will therefore concentrate in their gills a greater number of spermatozoa than can be accumulated in the same time by a smaller or less efficient mollusk. Present observations indicate, however, that no stimulating effect is to be expected if the number of spermatozoa in the water is below a certain minimum value. An inference can be made that to be effective the sperm must be present in sufficiently high concentration and that the proximity of oysters of both sexes on natural oyster bottoms is essential for the successful propagation of the species.

Immediately after spawning the female which may still contain a large number of ripe eggs becomes non-susceptible to further stimulation by sperm. This refractory period determined in a number of experiments was found to vary in different oysters from two to five days. Histological studies of the tissues of oysters preserved during this non-responsive period fail to reveal any conditions to which this state can be attributed. The ovaries contained a large number of ripe eggs lying free in the follicles and the oviducts appeared to be normal. In a short time the susceptibility is restored and the oyster can again be stimulated to spawn.

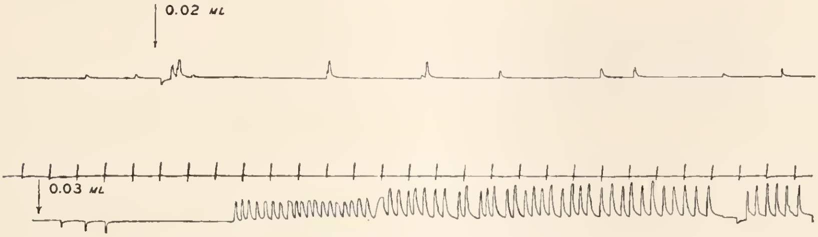

Fig. 4. Threshold concentration of sperm suspension in stimulating spawning reaction in *Ostrea virginica.* Vertical arrows indicate time when 0.02 ml. and 0.03 ml. of suspension were given. Time elapsed between the two tests, 1 hour. Middle line indicates one-minute intervals.

Temperature Requirement for Stimulation of Spawning by Sperm

In the experiments designed to determine the threshold temperature required for stimulation of spawning, ripe females were treated with sperm at 18° C. After the lapse of two hours, during which continuous kymograph records of shell movements were taken, the water was changed, warmed to 20.5° and the oysters given a second dose of the same sperm suspension. In several instances typical spawning reaction took place, indicating that the threshold temperature is between 18.6° and 20.5°. It is of interest to note that at 18° C. some of the females reacted to the presence of sperm by increased muscular contractions. The up and down excursions of the muscle were, however, very small and the reaction was not accompanied by the discharge of eggs. The rhythmicity of these small contractions, not observed under any other conditions, suggested an abortive spawning reaction which failed to reach its full development. Upon raising the temperature to 20.5° C. and adding a second portion of the same sperm suspension, oysters spawned in a typical manner. Similar conditions but differing only in the temperature levels, were observed in *O. gigas* which in one of the experiments at 22.5° responded to the addition of sperm by small rhythmical contractions (Fig. 5, upper line) and spawned one hour later immediately after the temperature was raised to 25° C. (lower line).

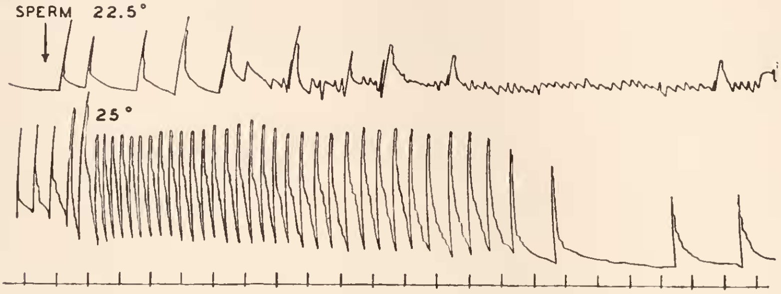

FIG. 5. Effect of temperature on the stimulation of spawning in *Ostrea gigas*. Abortive reaction at 22.5° (upper line); normal reaction at 25° C. Time interval, 1 minute.

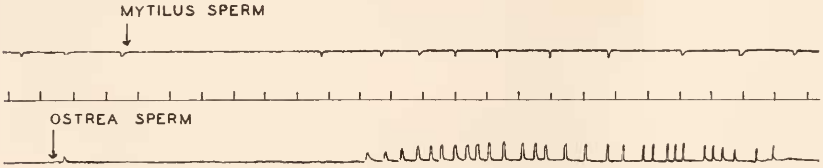

FIG. 6. Specificity of spawning reaction of *Ostrea virginica*. Time interval, 1 minute (middle line).

Can the temperature between 18.6° and 20.5° be regarded as critical for the spawning of *O. virginica?* In papers dealing with oyster spawning, the term "critical" is used in a very unqualified manner, without defining its exact meaning. Experimental evidence points to the fact that temperature around 20° causes no trigger-like effect on the spawning mechanism of the female. A large number of females capable of spawning were found to be unaffected by it and could not even be stimulated at this temperature by sperm. They required warmer water for the initiation of the spawning reaction. Thus we may conclude that the lower limit at which spawning may take place, i.e., between 18.6° and 20.5°, is not a critical temperature in the sense that it initiates ovulation and the discharge of eggs in all physiologically ripe females. Its significance is in the fact that, under laboratory conditions, below this threshold temperature stimulation by sperm is not effective. This conclusion is corroborated by the following observations showing considerable variation in the lower temperature at which spawning can be induced. In the experiments made on June 17, 1936, at 27.5°, sperm failed to provoke the spawning reaction in ripe females which, however, spawned 5 hours later at 33.5° C. Both times the same sperm suspension was used. Spawning was brief with a very light discharge of eggs. In another experiment (Aug. 28, 1934) sperm had no effect on ripe females at 22° C. but stimulation was effected one hour later at 25.6°. An inference from these observations is that sexual responses to chemical stimulation depend on the state of the female organism which may require higher or lower temperature for effectuation of the spawning reaction.[1]

TABLE III

Effect of temperature on the stimulation of spawning by sperm in Ostrea gigas

Date	Temperature	Spawning	Temperature	Spawning
July 17	27.7	–	30.0	+
July 22	22.6	–	30.0	+
July 22	22.6	–	30.0	+
Aug. 8	28.0	–	30.0	+
Aug. 12	28.0	–	30.0	+
July 7*	26.0	–	28.0	+

* Two oysters.

Similar conditions were observed also in *O. gigas*. In the experiments summarized in Table III, each female was given 1 ml. of sperm suspension and kept for one hour at the temperature indicated in column 2. Then the water was warmed to the degree shown in column 4 and a second dose of sperm was administered. Success or failure to spawn is indicated by plus or minus sign. The results clearly show that ripe females non-responsive to sperm at one temperature react very easily to the same suspension at higher temperatures. In more than 30 laboratory experiments carried out with *O. gigas* at Woods Hole no spawning could be induced by sperm at temperatures below 25° C. Yet under natural conditions Elsey (1936) succeeded in stimulating spawning in a large number of oysters of this species at temperatures between 19.4° and 20.2°. Hopkins in a personal communication to the author states that on September 9, 1931, a female *O. gigas* spawned in the laboratory tank in Olympia, Washington, at 16.8° C. The rôle of temperature in controlling spawning in *O. gigas* is not clear, but the observations just quoted indicate a rather wide range under which it may occur. There is a possibility that as the season advances ripe unspawned females become more responsive to stimulation and spawn at lower temperatures than at the beginning of the breeding period. This point requires, however, verification.

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[1] It has been found by Loosanoff that in June, 1938, oysters spawned in Long Island Sound at the temperature of about 17° C. The oyster larvae, however, failed to reach metamorphosis and perished. Some of these oysters taken by the author to the laboratory could not be stimulated to spawn at 20° C. and therefore the conditions which caused their early spawning in the Sound remained undetermined.

Specificity of Spawning Reaction

The question whether the spawning reaction of the female oyster can be stimulated by any other agents besides the sperm of the oyster and an increase in temperature was studied in a series of experiments in which sperm of various mollusks and several hormones were tested. The results were negative. Ripe females were entirely indifferent to the addition of live sperm of *Venus mercenaria*, *Mya arenaria*, *Mytilus edulis*, *Modiolus modiolus* and *Pecten irradians*, but immediately reacted to the addition of oyster sperm (Fig. 6). The injection of thyroidin, theelin, and other hormones which were found to stimulate spawning of the male [2] were entirely ineffective.

So far as chemical stimulation is concerned, the spawning reaction of the ripe female appears to be specific and as present experiments indicate can not be provoked by any other agent except the sperm of an oyster. This specificity is not restricted, however, to the sperm of the same species for it has been found (Galtsoff, 1931) that stimulation of spawning in the female *Ostrea virginica* can be induced by the sperm of *O. gigas*, and vice versa. In both cases typical reaction ensues. Previous experiments (Galtsoff and Smith, 1932) prove that eggs of both species can be cross-fertilized and that the hybrids develop normally. It would be of interest to determine whether spawning in *O. virginica* and *O. gigas* can be provoked by sperm of other species of oysters. Nelson (1931) reports that sperm of *O. angulata* stimulates *O. virginica*, while Galtsoff (1931), when working in Honolulu, was unable to demonstrate any effect of the sperm of *O. commercialis* [3] on *O. virginica* and vice versa. The opportunity to make similar tests with other species has not yet occurred.

The biological significance of the specificity of the spawning reaction is obvious. The oyster discharges its eggs only when the right sperm capable of fertilizing them is present in the water. A non-specific response would result in a waste of the discharged material and eventually lead to the cessation of propagation and the extinction of the species.

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[2] The experiments with males will be fully discussed in a separate paper.

[3] In the above-mentioned paper reference is made to *O. cucullata*. It has been found since that the specimens used in the experiments were *O. commercialis*.

Active Principle of Sperm Suspension

Attempts to determine the properties of the active principle of sperm suspension were made by testing its solubility in water and in various fat solvents and by ascertaining the effects of temperature and dehydration on its stability.

The first step was to determine whether live sperm is necessary to provoke spawning reaction. The question was answered in the negative by the experiments in which sperm killed by grinding with sand in sea water or in distilled water was used. Sand was purified by repeated washings in water, boiling in concentrated HCl, and washing again in distilled water until the disappearance of the last trace of acid. In killing the sperm the following procedure was used: A piece of ripe testis was ground for 30 minutes with ten times its volume of sand. Then a known quantity of filtered sea water was added, and the mass thoroughly stirred and set aside. As soon as the sand settled the supernatant suspension was pipetted and examined under the microscope. It contained no live sperm. Given to the ripe female kept in water at 23° C. it induced typical spawning, the latent period and the duration of which were within the normal range of the reaction stimulated by live sperm. Eggs discharged by the female were unfertilized and failed to develop during the following twenty-four hours. Identical results were obtained with sperm killed by grinding with sand in distilled water. The tests were repeated several times and always gave identical results which prove that killed sperm does not lose its stimulating power although its fertilizing ability has been destroyed.

The next step consisted in determining whether the active principle is present in the liquid phase of the sperm suspension or is located in the suspended particles. Filtration through tough Watman filter paper Nos. 44 and 52 failed to remove the spermatozoa and the filtrates were as effective in inducing spawning as the unfiltered standard suspension. Complete separation of sperm from the liquid phase has been accomplished by ultrafiltration, using ultrafine Zigmondy collodion membrane capable of retaining Congo Red particles. Samples of clear filtrates obtained in this manner were examined under the microscope and after being found free of suspended material were injected into the pallial cavity of the ripe females. After the lapse of one hour during which no spawning occurred, the residue was collected from the membrane and given to the oyster. As can be seen from Table IV, the filtrate lacked stimulating power, whereas the residue which still contained active sperm, was effective. In all four experiments eggs were fertilized and developed in a normal way.

It is concluded from the results of these experiments that the active principle of sperm suspension is not present in solution but is probably located in the bodies of spermatozoa. Unfortunately, a small number of blood cells and various elements of the connective and epithelial tissues are also present in the suspension. It is, however, unlikely that they are the bearers of the active principle, for it has been determined in a series of experiments that neither the blood nor the tissue juices of the male exert a stimulating effect on the female.

The insolubility of the active principle in the distilled water was demonstrated by ultrafiltering an extract prepared by grinding a piece of testis with sand. In these experiments 1.5 grams of ripe male gonad was minced in 25 ml. of distilled water and ground for 30 minutes in a glass mortar with 5 grams of purified sand. The sand was allowed to settle and a fine suspension was pipetted off and filtered through an ultrafine collodion membrane. The separate tests were performed with two ripe females kept at 20.9° and 21.5° C. Each time a fresh sample of gonad extract was prepared. The filtrates had no effect on the females which, however, reacted in a typical manner to the addition of the residue collected from the membrane. The discharged eggs were not fertilized and failed to develop. Identical results were obtained with sperm extracts in sea water. The experiments demonstrate that the active principle being insoluble in water remains within the cellular fragments of the gonad tissue from which it cannot be separated by this method.

TABLE IV

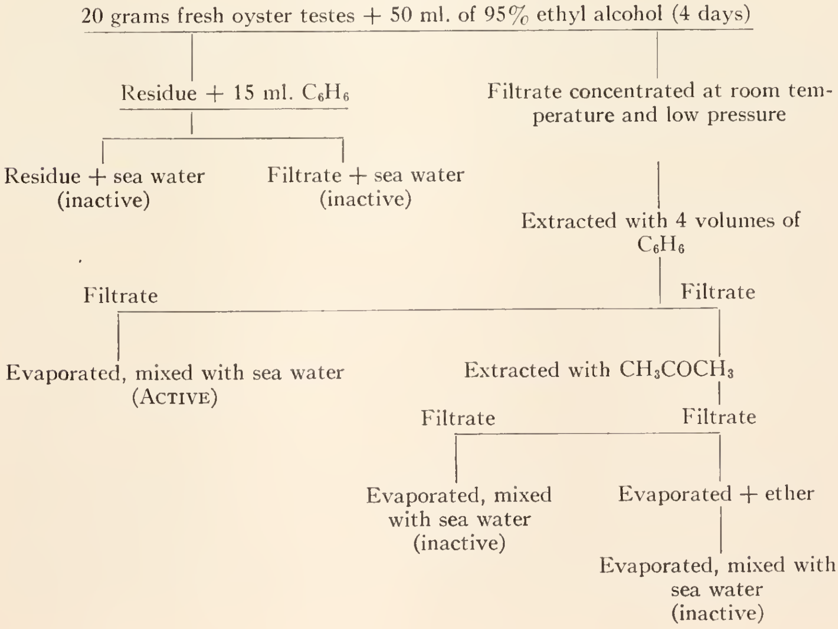
Effect of ultrafiltration on the stimulating power of sperm suspension

Date	Temp.	Membrane	Filtrate	Spawning	Residue	Spawning
	° C.		*ml.*		*ml.*	
June 19.......	23.8	Fine	2.5	–	1.0	+
June 29.......	26.8	Finest	2.5	–	1.0	+
July 21.......	25.0	Ultrafine	10.0	–	5.0	+
July 21.......	27.0	"	10.0	–	5.0	+

EXTRACT OF ACTIVE PRINCIPLE

Since the active principle of sperm suspension was found to be insoluble in water, attempts have been made to extract it in alcohol, ether, benzene, chloroform, acetone, and other fat solvents. Out of many tests, successful results were obtained by employing the method similar to that developed by Koch (1931) and Gallagher and Koch (1929) for the preparation of the male hormone. From 15 to 20 grams of testes of several ripe oysters, after being freed as much as possible from adjacent organs and connective tissue, were finely mashed in a glass mortar and extracted for four days at room temperature with about 50 ml. of 95 per cent ethyl alcohol. The alcoholic solution was filtered and the filtrate evaporated over night in vacuo until an aqueous suspension of lipins was obtained. Since some alcohol still was detectable by smell, about 15 ml. of pure benzene were added and the preparation evaporated almost to the consistency of paste. It was then extracted by repeated shaking with four volumes of benzene until the last benzene portion showed no color. The combined benzene portions were then evaporated to dryness under a vacuum and the extract, mixed with a small amount of filtered and boiled sea water, was injected into the pallial cavity of a ripe female capable of spawning. Out of five tests with this preparation four gave positive results. The reaction induced by the extract did not differ in any way from normal spawning reaction (Fig. 7). The latent periods varied between 10 and 20 minutes. The temperature of the water during the experiments fluctuated between 23.1 and 23.7° C. Attempts to purify the lipin mixture by dissolving it in acetone and separating the inert precipitate were not successful and all the fractions thus obtained had no specific action on females. Various steps of this process and results obtained with different fractions are shown in the following diagram.

Preparation of Sperm Extract


20 grams fresh oyster testes + 50 ml. of 95% ethyl alcohol (4 days)
Residue + 15 ml. C_6H_6
Filtrate concentrated at room temperature and low pressure
Residue + sea water (inactive)
Filtrate + sea water (inactive)
Extracted with 4 volumes of C_6H_6
Filtrate
Filtrate
Evaporated, mixed with sea water (ACTIVE)
Extracted with CH_3COCH_3
Filtrate
Filtrate
Evaporated, mixed with sea water (inactive)
Evaporated + ether
Evaporated, mixed with sea water (inactive)

In all the cases in which the preparation was found to be physiologically inactive, live sperm was given to the female and a positive spawning reaction was obtained, thus proving that the test oyster was capable of spawning. Small yields of active benzene extract precluded the possibility of further chemical analysis.

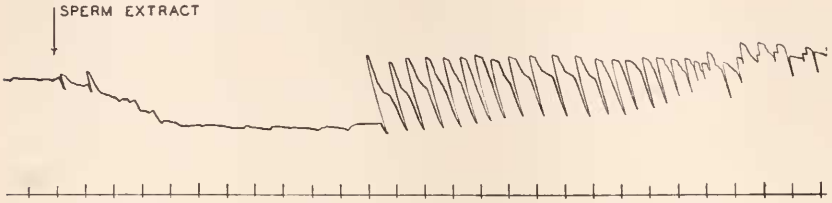


Fig. 7. Stimulation of spawning reaction in *Ostrea virginica* by the sperm extract. Time interval, 1 minute.

Instability of the Active Principle

The active principle is thermolabile and loses its power after a brief exposure to temperatures ranging between 54° and 57° C. This fact was established in a series of experiments summarized in Table V.

Table V

Effect of heating on stimulating power of sperm suspension. Second column indicates the temperature at which sperm was tested. Third and fourth columns indicate the temperature used in heating sperm suspension and time of exposure.

Date	Temp.	Heated sperm suspension			Spawning	Normal sperm suspension	Spawning
				Dose			
	° *C.*	° *C.*	*minutes*	*ml.*		*ml.*	
July 8....	25.2	54	15	2	–	2	+
July 9....	23.0	55	15	1	–	1	+
July 10....	23.5	56	15	1	–	1	+
July 11....	23.4	58	15	1	–	1	+
July 13....	23.0	55	15	1	–	1	+
July 13....	23.0	55	15	1	–	1	+

In each of these experiments standard sperm suspension was divided into two portions. One was heated for 15 minutes, counting from the time the desired temperature was reached. The second portion served as control and was administered one, or in some cases, two hours after the treatment with the heated sperm. It is clear from the examination of Table V that 15 minutes at a temperature of 54° and above suffices to destroy the potency of the active principle of sperm suspension.

Microscopic examination showed that all motion in the heated sperm had ceased and some of the spermatozoa were cytolized.

Sperm dried in the oven at 35° C. or at room temperature in vacuo completely lost its stimulating power and had no effect on ripe female oysters which, however, reacted to the addition of live sperm.

Probable Mode of Action of Sperm

Although the mode of action of sperm in stimulating spawning of the female is not known, certain speculation appears to be permissible. Our attention is first focused on the duration of the latent period which, as has been shown above, varies from 6 to 38 minutes. The length of time required for the realization of spawning is significant for it clearly shows the difference between this sexual reaction and an ordinary neuro-muscular response the latent period of which is measured in seconds. Furthermore, the duration of the latent period suggests that sperm may act through the digestive tract or after being absorbed by the gills and mantle of the oyster. The first possibility known to exist in *Platynereis megalops* (Just, 1914) has been investigated in the following manner. A large amount of fine carmine suspension was added to a number of actively feeding adult females. Every three minutes one of them was taken out, dissected and its stomach contents examined for the presence of carmine. It was found that at 22.6° C. a particle suspended in water reaches the digestive tract in about 7 to 12 minutes. It appears to be significant that the time required for the ingestion is about the same as that of the latent period of the spawning reaction. In another set of experiments attempts were made to prevent the sperm from reaching the digestive tract by plugging the mouth with beeswax and plastocele. Yonge (1928) used this method in a study of the absorption of glucose by *O. edulis.* In the present experiments Yonge's technique as well as several modifications of his original method was used without success. In all the cases, the females with plugged mouths spawned upon the addition of sperm. But the dissection of their stomachs revealed the presence of carmine particles which were added to the sperm suspension. This control measure proved the inefficiency of the method in blocking the entrance to the digestive tract. Since carmine particles were not prevented in reaching the mouth and stomach, it is reasonable to expect that sperm also reached the digestive tract. Thus, the experiments failed to throw light on the manner in which sperm acts on females. It is, however, obvious that in stimulating spawning the active principle of sperm does not act directly through the chemical receptors of the tentacles and mantle which, as a rule, respond to chemical or mechanical stimulation within a few seconds.

On the assumption that rhythmical contractions of the adductor may be stimulated by some substance liberated during ovulation and distributed by the organs of circulation, blood of a spawning female was collected and injected in the tissues of another ripe female. In the first set of experiments blood was obtained from females stimulated to spawn by the addition of sperm. In the second series, the use of sperm was avoided and blood was obtained from the females spawning under the influence of temperature. Altogether eight experiments were performed with negative results. The females did not respond to the injection of blood of the spawning females but each of them responded in a typical manner to the addition of sperm. Negative results were also obtained with the blood and tissue juices of the male injected hypodermically or added to the pallial cavity of the female. In preparing the tissue juices the gonads were of course carefully avoided. In all the experiments oysters spawned immediately upon addition of sperm. The results show that of the various tissues of the male organism, testis alone is effective in provoking spawning.

It has been shown in the first part of this investigation (Galtsoff, 1938) that the spawning reaction, once initiated, proceeds in a typical manner irrespective of the nature of the stimulus. Evidence presented in this paper clearly shows that the latent period and duration of spawning reaction are independent of the intensity of the stimulus (degree of temperature or concentration of sperm) and that the reaction proceeds according to the "all-or-none" principle. The rôle of the stimulus apparently consists in setting in motion a chain of reactions which, once started, follow their course and cannot be stopped or inhibited.

Rhythmical contractions of the adductor muscle, typical for spawning reaction, are not caused by the direct action of sperm on the neuroreceptors of the female. This is proven by the fact that spawned-out and unripe females fail to respond to the presence of sperm. On the other hand, the activity of the adductor during spawning cannot be considered as a simple ejection reaction caused by the presence of eggs in the pallial cavity, for the contractions often precede ovulation and sometimes continue after the latter is stopped. The peculiar behavior of the adductor muscle must be attributed to the internal impulses originating in the female organism during spawning. It is a specific sexual reaction of the female. As the breeding season advances, the intensity of the spawning reaction gradually decreases. Completely spent females are as a rule unable to respond to stimulation until the next breeding season. From a biological point of view, spawning reactions of the female oyster present an interesting example of specific physiological adaptation useful to a sedentary organism in providing better dispersal of eggs in the surrounding water and insuring greater chance for their fertilization. The advantages of the reaction in the survival of the species are obvious.

SUMMARY

1. Spawning of the physiologically ripe female *O. virginica* can be induced by exposing it to a constant temperature a few degrees higher than that of its environment or by quickly raising the temperature of the water from about 20° C. to 34–35° C. The second method, which appears to be more effective than the first one, gave positive results in 39.5 per cent of the tests.

2. Sperm of the oyster is a more powerful stimulant of sexual reaction for it easily induces spawning in oysters which do not respond to temperature stimulation. In the laboratory experiments, 55 per cent of the oysters which failed to react to increased temperature immediately responded to the addition of sperm.

3. The latent period of spawning reaction varies from 6 to 38 minutes and is independent of the concentration of sperm. A minimum concentration of sperm of about 100–150 spermatozoa per ml. of sea water is, however, necessary to induce a reaction.

4. The lowest temperature at which spawning in *O. virginica* was induced lay between 18.6° and 20.5° C. This threshold temperature is not, however, a critical temperature in the sense that it induces spawning or makes the stimulation by sperm effective in all physiologically ripe females. The lowest temperature level of the spawning reaction varies in different oysters depending on their responsiveness to stimulation.

5. The spawning reaction of *O. virginica* is specific in the sense that sperm of other genera of mollusks cannot provoke it.

6. The active principle of sperm suspension is thermolabile and insoluble in water. It can be extracted in 95 per cent ethyl alcohol and benzene.

7. The spawning reaction follows the "all-or-none" principle and once started cannot be inhibited or stopped. Rhythmical contractions of the adductor are considered to be due to impulses originating in the female organism during spawning. They are not ejection reactions caused by the presence of eggs in the pallial cavity.

8. The possibility of the action of sperm through absorption by the digestive tract or through the gills is discussed.

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